The first untranslated exon of the human tenascin-C gene plays a regulatory role in gene transcription

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Abstract The transcription of the human tenacin-C (TN-C) gene is directed by a single promoter. Here we demonstrate, in transiently transfected cells, that two distinct regions of the untranslated 179 bp-long exon 1 play antagonistic roles in transcriptional regulation: bases from 1 to 20 strongly increase the transcription of the reporter gene CAT directed by the human TN-C gene promoter, while bases from 79 to 179 significantly reduce this activation.

Key words: Tenascin-C; Gene transcription; Enhancer; Silencer

1. Introduction

Human tenascin-C (TN-C) is an extracellular matrix glycoprotein composed of six similar subunits joined together at their NH_2-terminus by disulfide bonds [1–4]. During development of various organs, human TN-C displays a 'site- and time-restricted' expression which is especially evident in the developing central nervous system [5]. In adult tissues, human TN-C expression transiently increases during wound healing [1–3] and neo-expression or a dramatic increase in its expression, as well as a striking modification in its RNA alternative splicing, have been reported in several tumors [4,6]. Furthermore, fetal calf serum and cytokines induce human TN-C expression in cultured cells [1–3].

The gene encoding human TN-C has been cloned, its structure elucidated [7,8], and a preliminary characterization of the sequences responsible for its transcription has been performed [7]. Human TN-C gene transcription is directed by a single promoter, and a relatively large intron (≥ 18 kb) separates the first untranslated exon (exon 1, 179 bp long) from exon 2 which contains the translation initiation [7,9]. By transient transfection of cells producing and non producing human TN-C, we previously demonstrated that the presence of the complete exon 1 plus a part of the first intron of the human TN-C gene strikingly enhances the chloramphenicol acetyl transferase (CAT) gene transcription directed by the human TN-C promoter in human TN-C producing SK-MEL-28 melanoma cells. Here we report on the role played by different regions of the first exon of the gene in transcriptional regulation.

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2. Materials and methods

2.1. Cell lines, human TN-C promoter-CAT constructs, and DNA transfection

SK-MEL-28 human melanoma, U87-MG human glioblastoma, hamster InR1-G9, and mouse NIH 3T3 cells were cultured as in [7]. The functional activity of the human TN-C gene transcriptional regulatory regions was analyzed utilizing the previously [7] described recombinant vectors pTN220CAT (from −220 to +79, here more precisely called pTN220 + 79/CAT) and pTN650CAT (from −650 to +79, here referred to as pTN650 + 79/CAT) and by constructing two series of new vectors in which different regions of the human TN-C promoter and the exon 1, obtained by PCR (using UlTma DNA polymerase from Perkin-Elmer, Norwalk, CT), were cloned into the polylinker of pCAT Basic (Promega, Madison, WI) [7]. In the first series, the 24-mer 5′ primers had a 5′ *Pst*I restriction site and initiated at base −220 [7]. The 28-mer 3′ primers, starting in its 5′ end at bases 1, 20, and 179 of the human TN gene, respectively, and containing a *Xba*I restriction site at its 5′ end, were used. In the second series, the 26-mer 5′ primers had a 5′ *Pst*I restriction site and initiated at base −650 [7]. The 28-mer 3′ primers utilized were the same described above. The fragments thus obtained were cloned into pCAT-Basic vector, thereby creating the constructs pTN220/CAT, pTN220 + 20/CAT, pTN220 + 179/CAT, pTN650/CAT, pTN650 + 20/CAT, and pTN650 + 179/CAT, respectively. The promoterless pCAT-Basic and a construct in which 220 bp of the promoter and 79 bp of the exon 1 were cloned into pCAT-Basic with opposite orientation in respect to the transcription initiation site (pTNRev-CAT) were the negative controls.

Transient transfection by either the calcium phosphate or the DEAE-Dextran technique, CAT, and β-galactosidase activity assays were carried out as reported in [7]. DNA sequence analysis and alignements were performed using the GeneJockey (Biosoft, Cambridge, UK) and GeneWorks 2.3.1 (IntelliGenetics Inc., Mountain View, CA) softwares for Macintosh Power PC computers.

2.2. Reverse transcription and PCR amplification of total RNA (RT/PCR)

Total RNA was prepared from U87-MG and InR1-G9 cells transfected with the different CAT plasmids by the guanidine/cesium chloride method [10]. RNA preparations were subjected to a 30 min treatment with RNAse-free DNAse (RQ1 by Promega). The reverse transcription and the amplification by polymerase chain reaction (RT/PCR) of the DNAse-treated RNA was performed as described in [11]. CAT sequences were amplified using the following primers: *primer CAT1* 5′-TCAATGTACCTATAACCAGACC-3′ (from base 2401 to base 2422 in the plasmid pCAT-Basic) and *primer CAT2* 5′-AGGTTTCACCGTAACACGCCA-3′ (from base 2706 to base 2685 in the plasmid pCAT-Basic) giving an amplification product of 306 bp. The MHC class II-related gene β_2-microglobulin was used as an internal control to normalize CAT transcript levels measured by RT/PCR as previously detailed [11]. In order to identify possible amplification products generated by contaminating genomic or plasmid DNA, we subjected RNA preparations to PCR utilizing two sets of primers designed to amplify either the region from −220 to 1 of the human TN-C gene or the CAT gene. The analysis and quantitation of RT/PCR products was performed as described in [11]. To optimize RT/PCR, preliminary dose- response experiments were performed to determine the range of RNA concentrations at which PCR amplification was linear for each target molecule in either U87-MG or InR1-G9 cells as described in [11]. 0.5 ng and 250 ng of RNA of both cell lines were used

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in RT/PCR experiments for quantification of both CAT and β_2-microglobulin, respectively.

3. Results and discussion

Aim of this study was to investigate the transcriptional role of the human TN-C exon 1.

Alignment of human TN-C exon 1 with either rat or chicken exon 1 sequences reveals overall identities of 74% and 50%, respectively. As presented in Fig. 1A and E, a region with the highest homology between human and mouse exon 1 is present in the first 50 bp of exon 1. Similarly, human and chicken exon 1 sequences, although more discordant, present the highest homology in the 45 bp closest to the transcription start site (Fig. 1, panel B). However, the dot matrix plots presented in panels C and D of Fig. 1 clearly indicate that the homology between human, mouse, and chicken is significantly higher in TN-C exon 2 than in exon 1. This high level of homology extends over the entire coding sequences (data not shown). Copertino et al. [12], on the basis of sequence comparison between the mouse

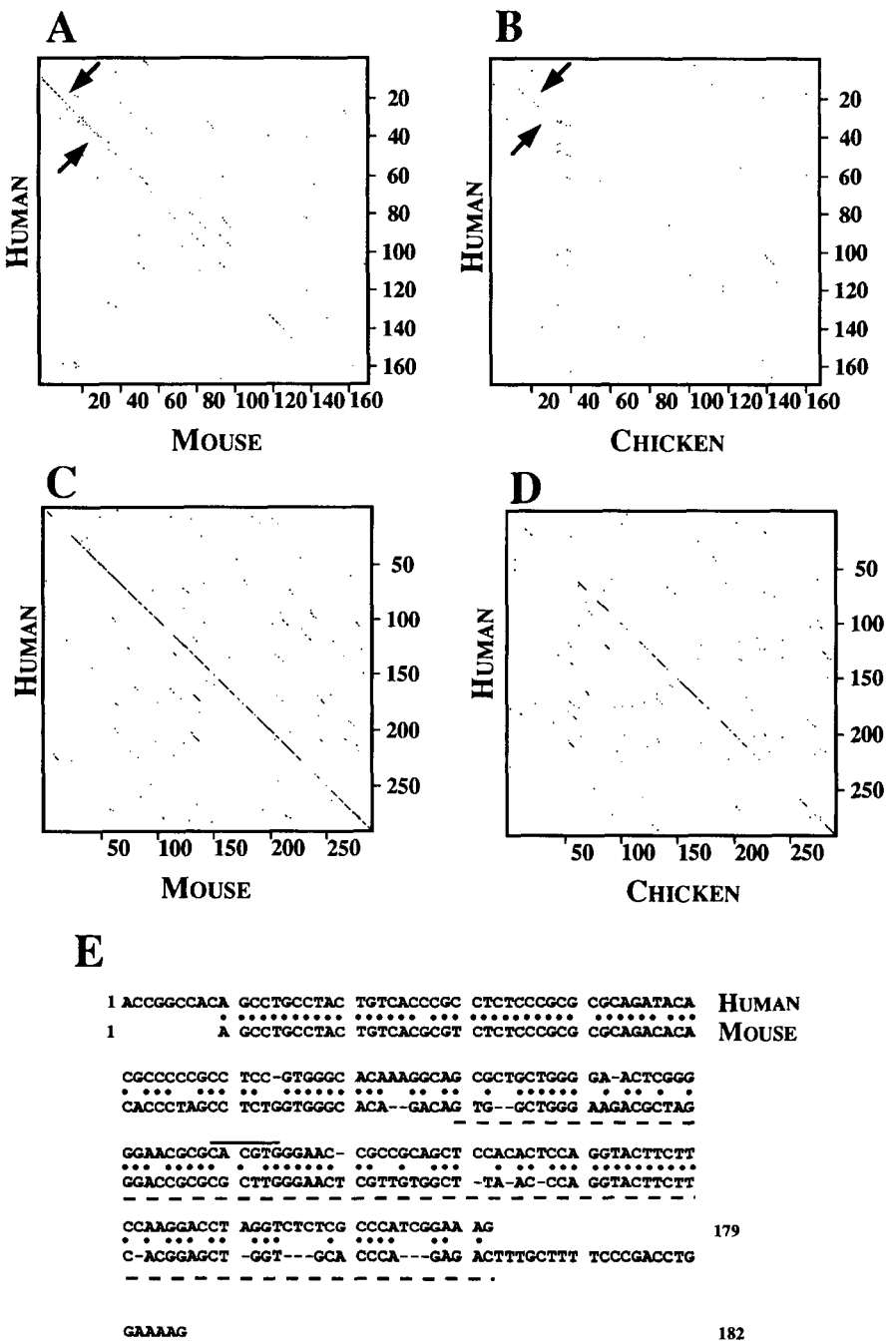

Fig. 1. Similarities of the human, mouse, and chicken TN-C, exon 1 and exon 2. The sequences of the entire exons 1 (A and B) and of the most 5′ 300 bp of exons 2 (C and D) of TN-C genes were compared using a dot matrix program. In the matrix, each dot represents 7 identities per 10 nucleotides. Regions of homology are indicated by slanted arrows. In panel E, the sequences of human [7] and mouse [12] TN-C genes are aligned. The dashed line indicates the region Si2 of the human exon 1 while the solid line covers the 'E-box'.

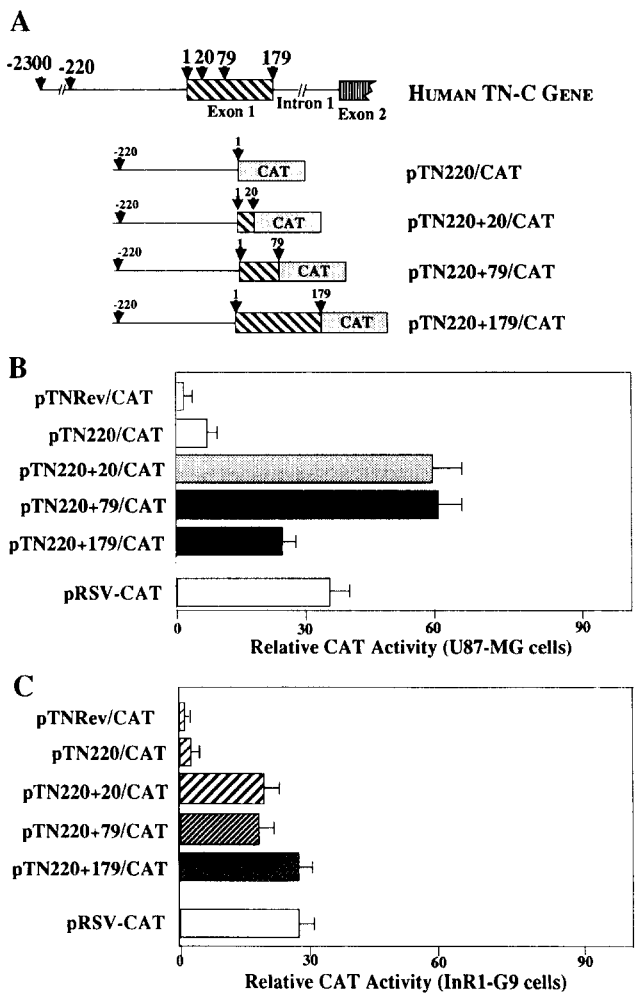

Fig. 2. Analysis of the the transcriptional properties of the human TN-C exon 1 by transient transfection of CAT reporter plasmids in U87-MG and InR1-G9 cells. In panel A, the structure of the 5′ region of the human TN-C gene as well as the transfected promoter/exon 1-CAT plasmids are schematically represented. U87-MG human glioblastoma (panel B) and InR1-G9 hamster glucagonoma (panel C) cells were transiently transfected with 12 μg of pRSV-CAT or each of the indicated recombinant plasmids and 2 μg of pCMV-β-Gal using the calcium phosphate or the DEAE-Dextran technique, respectively (as detailed in section 2). 48 h after transfection, cells were harvested and CAT and β-galactosidase activities in cell lysates were measured. Chloramphenicol acetyltransferase activities were normalized taking into account β-galactosidase activities measured in all the experiments. The average (±S.E.M.) of six independent experiments performed in duplicate for each cell line is presented.

and the chicken TN-C gene promoters, postulated that conserved regions (such as the proximal promoter, ~250 bp long) play a constant role throughout different species, while divergent regions imply species-specific temporal and spatial differences in the gene expression pattern.

To investigate whether different regions of human TN-C exon 1 display any specific transcriptional properties, a number of exon 1 deletion constructs were cloned into a CAT expression vector, and sequenced to confirm their identity (see section 2 and Fig. 2). The resulting plasmids, together with pCMV-β-Gal as an internal control for transfection efficiency, were transiently transfected into U87-MG human glioblastoma, SK-MEL-28 human melanoma, InR1-G9 hamster glucagonoma cells, and in NIH 3T3 mouse fibroblasts. Cell lysates were then assayed for both CAT and β-galactosidase activities. As illustrated in Fig. 2, a vector in which CAT expression is directed by the 220 bp immediately 5′ to the putative transcription start site is transcriptionally active in both human and rodent cell lines (from 5- to 10-fold over the negative control pTNRev/CAT). The addition to this proximal promoter of 20 bp of the exon 1 strongly increases the reporter gene expression in human and rodent cells (Fig. 2 and data not shown). This finding is in agreement with data reported by Pan and Greenblatt [13]. These authors demonstrated that mutations in nucleotides close to the transcription start site (either at its 5′ or at its 3′ side) influence the transcript elongation by affecting the interaction with selected components of the general transcription machinery [13].

The presence of a further 59 bp of exon 1 (pTN220 + 79/CAT) did not affect the ability of the human TN-C promoter to drive CAT gene transcription in both human and rodent cell lines (Fig. 2 and data not shown). The construct pTN220 + 179/CAT was 60% less efficient than pTN220 + 20/CAT or pTN220 + 79/CAT to drive the expression of the reporter gene

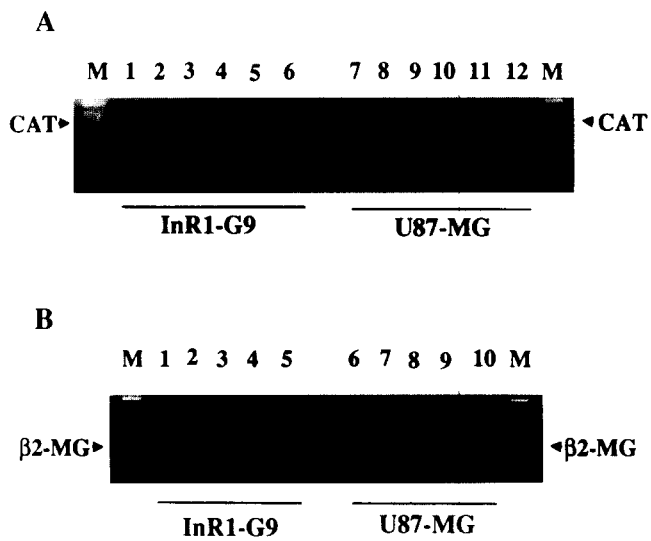

Fig. 3. RT/PCR analysis of CAT and β_2-microglobulin transcripts in U87-MG and InR1-G9 cells. Subconfluent cultures of U87-MG and InR1-G9 cells were transiently transfected with the following CAT recombinant expression vectors using the calcium phosphate or the DEAE-Dextran technique, respectively: none (lanes 1 and 7 in A); pTNRev/CAT (lanes 2, and 8 in A; 1 and 6 in B); pTN220/CAT (lanes 3, and 9 in A; 2 and 7 in B), pTN220 + 20/CAT (lanes 4, and 10 in A; 3 and 8 in B); pTN220 + 79/CAT (lanes 5, and 11 in A; 4 and 9 in B), pTN220 + 179/CAT (lanes 6, and 12 in A; 5 and 10 in B). After 48 h, cultures were extensively washed with PBS and harvested. Total RNA was prepared and subjected to RT/PCR as detailed in section 2. RT/PCR products were electrophoresed and agarose gels stained with ethidium bromide and photographed. The bands corresponding to CAT and β_2-microglobulin transcripts in ethidium bromide-stained gels were densitometrically scanned and the relative absorbances (expressed in arbitrary units) of two independent experiments (including that presented in the figure) are: CAT 9.0 (lane 2), 8.6 (lane 3), 25.2 (lane 4), 23.1 (lane 5), 20.4 (lane 6), 18.5 (lane 8), 43.7 (lane 9), 99.5 (lane 10), 111.2 (lane 11), 25.5 (lane 12); β_2-microglobulin 63.4 (lane 1), 29.0 (lane 2), 51.0 (lane 3), 38.1 (lane 4), 15.5 (lane 5), 98.4 (lane 6), 69.2 (lane 7), 124.0 (lane 8),82.0 (lane 9), 99.1 (lane 10).

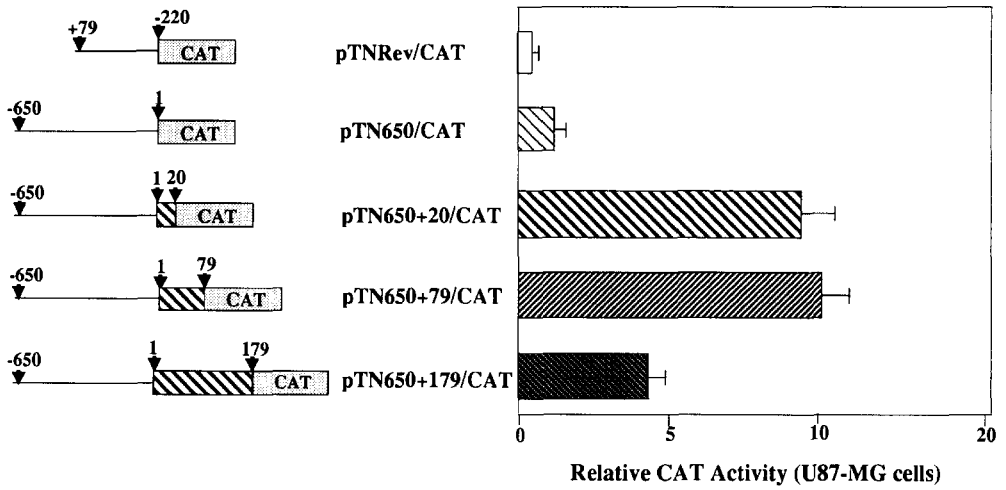

Fig. 4. The transcriptional properties of the human TN-C exon 1, analyzed by transient transfection of CAT reporter plasmids in U87-MG, do not depend on the size of TN-C promoter. On the left, the structure of the transfected promoter/exon 1-CAT plasmids are schematically represented. U87-MG human glioblastoma cells were transiently transfected with 12 μg of each of the indicated recombinant plasmids and 2 μg of pCMV-β-Gal using the the calcium phosphate technique, respectively (as detailed in section 2). 48 h after transfection, cells were harvested and CAT and β-galactosidase activities in cell lysates were measured. Chloramphenicol acetyltransferase activities were normalized taking into account β-galactosidase activities measured in all the experiments. The average (±S.E.M.) of four independent experiments performed in duplicate for each cell line is presented.

in human U87-MG (Fig. 2) and SK-MEL-28 (not shown) cells. However, the transcriptional activity of pTN220 + 179/CAT was similar to that of pTN220 + 20/CAT and pTN220 + 79/CAT constructs in transfected hamster InR1-G9 cells (Fig. 2) and murine NIH 3T3 cells (not shown). These data suggest that the regulatory element(s) interacting with the exon 1 sequence from +79 to +179 may be species-specific. This hypothesis is supported by the observation of the low sequence homology between different species in this region of TN-C exon 1 (Fig. 1).

The possibility existed that the regulatory elements present in the sequence from 79 to 179 of the human TN-C exon 1 were transcriptionally effective only in the context of the 220 bp-long proximal promoter. To verify this hypothesis, we transiently transfected cells with CAT expression vectors containing 650 bp of the human TN-C promoter instead of 220 bp. As depicted in Fig. 4, this set of vectors also revealed that the regions from 1 to 20 ('enhancer 1'; En1) and from 79 to 179 ('silencer 2'; Si2) of exon 1 exert positive and negative actions on transcription, respectively in human U87-MG and SK-MEL-28 cells (not shown). As expected on the basis of our previous results [7], the transcriptional activity of the 650 bp promoter constructs was remarkably lower than the activity of 220 bp promoter constructs.

The location of elements En1 (nucleotides 1–20 in exon 1) and Si2 (nucleotides 79–179 in exon 1) in a transcribed region of the gene raises the possibility that they regulate gene expression at the level of translation rather than transcription. Therefore, we determined through quantitative RT/PCR the levels of CAT mRNA in transiently transfected cells. As presented in Fig. 3, the RT/PCR results were parallel to those obtained from the CAT assays. This finding indicates that the two regulatory elements in the human TN-C exon 1 act by regulating the transcription of the reporter gene CAT.

The sequence analysis of exon 1 revealed, at nucleotides 106–111, the presence of an 'E box' (CACGTG), a previously reported consensus binding site for proteins belonging to the basic helix-loop-helix family of transcription factors (such as Myc and Max) [14,15]. It is noteworthy that the sequence homology between human TN-C exon 1 and Myc-Max DNA binding sites, like those detectable in prothymosin-α and in ornithine decarboxylase genes [16,17], extends significantly upstream and downstream of the 'E box' (Fig. 1E). In a recent paper Shor et al. reported that two Myc-Max binding motifs constitute the attenuator element Attenuator 1 responsible for RNA elongation arrest in the murine ornithine decarboxylase gene [18]. However, the 'E-box' is not present in the sequence of mouse TN-C exon 1 [12]. This observation suggests that this element is not important for TN-C transcriptional regulation or, alternatively, that differences exist between the regulatory mechanisms responsible for TN-C gene transcription in the two species.

Transcribed DNA sequences are usually excluded from reporter constructs used to characterize promoters. This leads to the possibility that transcriptional regulatory elements present in exons of several genes go undetected. In the last few years, a growing number of examples of exonic and intronic transcriptional regulatory sequences, either positive [19–23] or negative [21–26], have been reported.

Both chicken and mouse tenascin-C gene transcription has been postulated to be negatively regulated by sequences placed upstream to the approximately 250 bp long promoter [12,27]. These results are consistent with our previous observations in the human gene [7]. The present results demonstrate that additional positive and negative elements are present in the transcibed region of the human TN-C gene.

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